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Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

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Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

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Blatt 2 der Bescheinigung
Sheet 2 of the certificate
Page 2 de l'attestation

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A protein hydrolysate rich in di- and tripeptides

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Protein hydrolysate rich in di-and tripeptides

Field of the invention

The present invention relates to protein hydrolysate and the uses thereof.

Background of the invention

Protein hydrolysates enjoy increasing interests for both medical and non-medical applications. In both applications an easily assimilable diet featuring facilitated gastrointestinal uptake of proteins is a factor of prime importance. Protein hydrolysates for medical applications also require strongly reduced allergenic properties; for products intended for non-medical applications, good taste characteristics and good solubilities under acid conditions are important extra's. Unfortunately the hydrolysis process required to realise these benefits comes with a number of disadvantages. Well known are bitter off-tastes, residual immunogenic materials, low yields of nutritionally indispensible amino acids, high osmotic values caused by the release of free amino acids and, finally, limited acid stabilities.

In prior publications several enzyme mixtures aimed at optimising hydrolysate characteristics and lowering production costs have been described. All of these publications refer to the use of single or mixed endoproteases. Examples include EP 321 603, which refers to the use of animal-derived endoproteases like trypsin, chymotrypsin and pancreatin, and EP 325 986 and WO 96/13174, which favor the use of endoproteases obtained from *Bacillus* or *Aspergillus* species. Unfortunately these enzyme combinations always yield peptide mixtures which are bitter and exhibit a broad molecular weight distribution. Large molecular weight peptides are undesirable because they are responsible for the allergenic response and their uptake requires additional enzymatic processing steps in the intestine. Reducing the bitter off-taste in the hydrolysates makes the use of exoproteases such as aminopeptidases or carboxypeptidases indispensible. Disadvantages of this debittering process are the release of substantial quantities of free amino acids and thus brothy off flavors and losses of nutritionally important amino acids.

In conclusion one can say that the industrial production of protein hydrolysates continues to rely on enzyme mixtures which are far from optimal so that expensive purification steps are needed to produce peptide mixtures with a sub-optimal size distributions.

Under normal conditions the proteins present in food are gradually hydrolysed to smaller fragments which are finally transported across the wall of the small intestine. During passage through the gastro-intestinal tract a number of different proteases that originate in the stomach, pancreas and small intestine are active. Endoproteases such as pepsin, trypsin and chymotrypsin cleave the large molecular weight proteins into smaller oligopeptides. These oligopeptides are then further hydrolysed by a number of other enzymes such as di- and tripeptidyl peptidases as well as amino- and carboxypeptidases. Then the final steps in the hydrolysis take place in the small intestine to result in a mixture of free amino acids and di- and tripeptides (Grimble,G.K. 1994. Annu. Rev. Nutr. 14;419-447). It is likely that especially peptides that resist further proteolytic hydrolysis form a major fraction of the surviving population of di- and tripeptides. It has, for example, been reported that di- and tripeptides carrying carboxyterminal proline residues exhibit stabilities in the body which are upto 3 orders of magnitude higher than other peptides (Ashmarin,I.P. *et al.;* Biochemistry (Moscow), Vol 63, No 2,1998, pp119-124). Carrier systems specific for the transport of either the free amino acids or the di- and tripeptides present are responsible for the efficient transport across the intestine wall. A peptide sequence-independent mechanism capable of transporting quantitatively significant amounts of intact di- and tripeptides has been identified (Doering, F. *et al; 1998;* J. Biol. Chem. 273,23211- 23218). After entering the blood circulation, the peptides may act as potential physiological modulators of metabolism. The physiological effects of peptides with opioid, ACE-inhibitory as well as antithrombosis, antiulcer, antiarthritic and anorectic activities have been described (Pihlanto-Leppala, A; Trends in Food Science & Technology 11 (2001) 347-356; Ashmarin, I.P. *et al.;* Biochemistry (Moscow), Vol 63, No 2,1998, pp119-124).

The recent commercialisation of various protein hydrolysates claiming antihypertensive effects emphasize the scope of protein hydrolysates containing such "bioactive" peptides in medical and non-medical applications.These bioactive peptides and protein hydrolysates containing such peptides have been described in a number of patent applications. For example, WO9700078 describes hydrolysates obtained by incubation with probiotic bacteria

or enzymes obtained from such bacteria, WO9916461 describes the inhibition of angiotensin-converting enzyme by specific tripeptides obtained by fermentations of Lactobacillus, WO200132905 describes the preparation of a product containing antihypertensive peptides by fermenting casein with lactic acid bacteria. Several other applications describe the use of highly purified or chemically synthesized peptides for reducing blood pressure or treating diabetes, renal impairment or obesity.

Description of the invention

The present invention provides a process to produce protein hydrolysate which is rich in di- and/or tripeptides whereby preferably the peptides are rich in proline at one end of the peptide preferably the peptide has a carboxy terminal proline. Preferably the protein hydrolysate of the invention is non-bitter.

According to a preferred embodiment of the process of the invention, the protein or proteinaceous substrate selected is contacted with a suitable proline specific endoprotease (PSE). Moreover this substrate is contacted with a dipeptidase (DPAP) and/or suitable tripeptidase (TPAP). Advantageously the protein substrate is pre-fermented before contacting with PSE. This pre-fermentation can be an incubation with endoprotease, such as a serine or metalloendoprotease to partly hydrolyse the protein. We have found that both PSE and the DPAP and TPAP are in general more effective on such prehydrolysed protein substrates

The protein substrate or the partial hydrolysate formed can first be subjected to the PSE and subsequently DPAP and/or TPAP can be added. Especially in case the optimal activity conditions of the enzymes differ, a two step process may be preferred.

To be useful as processing aids in the preparation of food ingredients, an enzyme preferably meets a number of strict economical and legislative criteria. To meet the legislative criteria the enzyme should be obtained from an unsuspect source, for example a food-grade microorganism. To meet the economical criteria, the enzyme should be producible in high yields and exhibit a number of biochemical characteristics such as a long term stability under industrial processing conditions. To minimise the risks of microbial infections under such non-sterile conditions, industrial processing often implies acidic pH

conditions and a temperature of 50 degrees C or higher. An enzyme used in the present invention advantageously meet these demands.

The present invention further provides a hydrolysate rich in di- and/or tripeptides whereby preferably these di- and/or tripeptides are rich in carboxy terminal proline. Rich in di and/or tripeptides means that at least 20 molar%, preferably at least 25molar%, more preferably at least 30 molar% of the hydrolysate is present as di and/or tripeptide. Rich in proline means that at least 20%, preferably at least 30%, more preferably at least 40% and even advantageous 50% of the proline present in the starting protein substrate, is present in the di- and/or tripeptides preferably present as carboxy terminal proline in the di- and/or tripeptides. Preferably 30% of the tripeptides, more preferably 35% of the tripeptides have a carboxy terminal proline residue.

Although the main outlets for protein hydrolysates are infant formula and food products for hospitalised persons, products intended for persons with non-medical needs, such as athletes or people on a slimming diet form a rapidly growing segment. Whey protein represents a very suitable substrate for producing hydrolysates for the various applications. Whey protein is relatively rich in socalled "essential" and "branched chain" amino acids and has a high biological digestibility. Moreover, whey hydrolysates exhibit relatively low bitterness profiles.

In comparison with whey, proteins like casein, wheat gluten, soy, rice protein, chicken feathers and gelatin exhibit vastly different amino acid compositions. On the basis of their amino acid composition, some of these proteins form potentially the substrate of choice for the production of hydrolysates for niche markets. For example, wheat gluten is extremely rich in glutamine and rice protein is rich in arginine residues. Both amino acids are known to improve physical endurance and the recovery from high intensity exercise. However, as a free amino acid glutamine is not stable so that supply in a readily assimilable peptide is advantageous. Chicken feathers form a cheap and potentially important source of cysteine, an amino acid with an important role in modulating immune functions and fighting oxidative stress. Like glutamine, cysteine is a labile compound that is preferably supplied in the form of di-or tripeptides. However, upto the present invention the development of optimized hydrolysis protocols for such niche products is economically not viable.

Casein, gelatin and wheat gluten all contain high levels of proline residues. As mentioned before, proline confers an increased stability to peptides hereby increasing their potential significance in eliciting physiological effects. However, upto now peptide bonds involving proline residues are notoriously difficult to cleave so that hydrolysates prepared from these substrates contain major fractions of large molecular weight material. Moreover, proline represents a very hydrophobic amino acid and yields extremely bitter hydrolysates. Thus, using existing technologies the production of acceptable hydrolysates from such proline-rich substrates would lead to low yields and highly priced products.

The implication is that commercially attractive hydrolysates featuring high proportions of tripeptides are not available, certainly not if the hydrolysate is obtained from a proline-rich substrate. The present invention discloses an enzyme mixture that would permit simple protocols to convert all relevant proteinaceous substrates into hydrolysates with a good taste, an efficient gastrointestinal uptake and, if required, a high content of bioactive peptides, which is highly desirable.

In all of these applications protein hydrolysates offer attractive advantages such as lowered allergenicities, facilitated gastro-intestinal uptake, less chemical deterioration of desirable amino acids like glutamine and cystein and finally, absence of proteinaceous precipitations in acid beverages during prolonged storage periods. All these advantages can be combined if the hydrolysate is offered as di- and/or tripeptides or a mixture thereof. To obtain the desired mixture of di- and/or tripeptides, a hydrolysis process involving a combination of various di- and/or tripeptidylpeptidases can be used. According to the invention several of highly useful di- and tripeptidylpeptidases are preferably used in a relatively pure state. Preferred are those di- and/or tripeptidylpeptidases that exhibit a low selectivity towards the substrate to be cleaved, i.e. exhibit minimal amino acid residue cleavage preferences only. Preferred are combinations of those di- and/or tripeptidylpeptidases that hydrolyse high percentages of the naturally occurring peptide bonds. Despite this high activity to naturally occurring peptide bonds, a total hydrolysis to free amino acids is prevented by the nature of the di-and/or tripeptidylpeptidases. Also preferred are those di- and/or tripeptidylpeptidases that are optimally active between pH 4 to 8 and exhibit adequate temperature stability. Adequate temperature stability implies that at least 40%, preferably at least 60%, more preferably between 70 and 100% of the initial

hydrolytic activity survives after heating the enzyme together with the substrate for 1 hour at 50 degrees C.

Tripeptidyl peptidases or tripeptidases are enzymes that can release tripeptides from either the N-terminus of the oligopeptide (" tripeptidyl aminopeptidases") or from the oligopeptide's carboxyterminus ("tripeptidyl carboxypeptidases"). The various physiological advantages of the mixture of tripeptides formed by such enzymes was illustrated above. Tripeptidyl aminopeptidases (EC 3.4.14) have been isolated from mammelian as well as plant sources. Microorganisms from which tripeptidylpeptidases have been isolated are for example *Streptomyces* species (JP08308565, WO 9517512 and US 5856166)), *Porphyromonas gingivalis* (WO 200052147), *Dictyostelium discoidum* and *Aspergillus* species (WO 9614404). To date the occurrence of tripeptidyl carboxypeptidases (EC 3.4.15) has been demonstrated in mammelian cells and in microorganisms for example *Clostridium histolyticum*.

Especially a mixture of a di and/or tripeptidyl amino peptidase and a di and/or tripeptidyl carboxy peptidase are preferred in the process of the present invention. We have found that such a mixture can decrease the reaction time. Moreover, a higher amount of di and tripeptides is formed if compared with the use of a single amino or carboxy di or tripeptidyl peptidase. Tripepdidylpeptidases especially suitable in the present invention are described in our co pending patent application PCT/EP02/01984. These enzymes are obtained from *A. niger*. In table 1 of PCT/EP02/10984 the seq ID number of di and tripeptidases are given, whereas the corresponding sequences are given as well in this application.

Main aim of the hydrolysates is to minimize the allergenicity of the product or to facilitate gastro-intestinal uptake. In the production of such hydrolysates the use of dipeptidyl- and/or tripeptidyl-peptidases is of special importance as these offer an efficient way for producing hydrolysates.

The enzyme mixture according to the invention is characterised in that it may comprise another endoprotease such as a serine protease or a metallo endoprotease in conjunction with a proline-specific endoprotease (E.C. 3.4.21.26) which work together to provide a primary protein hydrolysate. Optionally the proteinaceous substrate may be predigested with an endoprotease preferably a serine or metalloendoprotease and

subsequently, after the inactivation of the endoprotease, exposed to the proline-specific endoprotease.

Serine proteases represent a well known class of alkaline endoproteases and some of its most important representants such as subtilisin (E.C. 3.4.21.62) and chymotrypsin (E.C. 3.4.21.1) prefer cleavage of the peptide chain at the carboxy terminal side of hydrophobic amino acids such as Tyr, Trp, Phe and Leu. The enzyme mixture of the invention may contain chymotrypsin and/or subtilisin. Subtilisin is produced by species of *Bacillus*, has a particularly broad substrate specificity and a broad, alkaline pH optimum. The enzyme is optimally active between 50°C and 60°C. The enzyme is cheaply available as a regular commercial product and is useful in the production of, for example, various milk hydrolysates. Chymotrypsin may be obtained from animal pancreas, has a somewhat narrower substrate specificity at slightly more alkaline pH values than subtilisin and is optimally active below 50 degrees C.

The class of metallo endoproteases is wide spread in bacteria, fungi and higher organisms.They can be separated into the neutral and acid metalloproteases. Of these two subclasses only the neutral proteases exhibit the desirable cleavage preference i.e. cleaving the peptide chain on the carboxy terminal side of hydrophobic amino acid residues such as Phe and Leu. Well known representants of the category of the neutral metallo proteases are bacillolysin (E.C. 3.4.24.28) and thermolysin (E.C. 3.4.24.27) and either, or both of these, may be present in the enzyme mixture of the invention. Both enzymes are obtained from *Bacillus* species and exhibit maximum activity under neutral or slightly alkaline conditions. Less well known representants of these neutral metallo endoproteases have been obtained from *Aspergillus* species. In those cases in which the proline specific endoprotease is not used for its debittering effects but to aid in the hydrolysis of proline rich protein sequences, combinations with the class of the acid metalloproteases, as for example deuterolysine (EC 3.4.24.39) can be advantageous

Our co pending patent application PCT/EP01/14480 describes the use of a proline–specific endoprotease which, in conjunction with the prior art endoproteases, is able to generate non-bitter protein hydrolysates. This proline-specific endoprotease is an enzyme capable of cleaving peptides or polypeptides at the carboxy-terminal end of proline residues.

Proline-specific endoproteases are widely found in animals and plants, but their presence in microorganisms appears to be limited. To date, proline-specific endoprotease have been identified in species of *Aspergillus* (EP 0 522 428), *Flavobacterium* (EP 0 967 285) and *Aeromonas* (J.Biochem.113, 790-796), *Xanthomonas* and *Bacteroides*. We have shown that a high incidence of proline residues at the carboxy terminal end of peptides can be correlated with low bitterness. Moreover we have demonstrated that the desired high incidence of carboxy terminal proline residues can only be achieved with high concentrations of a proline-specific endoprotease , i.e. concentrations that exceed the activity specified in JP5015314 by several orders of magnitude and moreover in the absence of a carboxypeptidase.

In conjunction with prior art endoproteases, the proline-specific endoprotease is capable of extensively hydrolysing proline-rich proteins yielding relatively small peptides with a narrow size distribution. Because of the cleavage preference the proline-specific endoprotease, many of the peptides formed have a carboxyterminal proline residue. Furthermore the processing of the hydrolysate is relatively simple as a debittering step by exoproteases is not involved so that only low levels of free amino acids will be formed.

From an economic point of view the implication of this observation is that there exists a clear need in the present process for the use of proline-specific endoproteases in high quantities and a relatively pure form, which is described in our co pending application PCT/EP01/14480. A preferred way of obtaining purified and isolated PSE is via the overproduction of such a proline-specific endoprotease using recombinant DNA techniques. As many food products are acidic and long term enzyme incubations under industrial, non-sterile circumstances require acidic incubation conditions and a processing temperature of 50 degrees C or higher to prevent microbial contamination, a more preferred way of doing this is via the overproduction of an acid stable proline-specific endoprotease using recombinant DNA techniques. A particulary preferred way of doing this is via the overproduction of an *Aspergillus* derived proline-specific endoprotease and a most preferred way of doing this is via the overproduction of an *Aspergillus niger* derived proline-specific endopeptidase.

Once the new enzyme has been made available in a relatively pure form, other new and surprising applications are envisaged which have technical and economical advantages.

A new application would be the creation of non-bitter hydrolysates from proteinaceous substrates with novel amino acid compositions. Such novel amino acid compositions may offer serious benefits in certain food and medical applications. Examples are casein or wheat gluten or maize protein isolate with high levels of hydrophobic amino acid residues and, more specifically, proline residues present. Hitherto such substrates were of no practical use because of the objectional bitter tastes generated upon hydrolysis and the limited degrees of hydrolysis obtained using prior art methods. Using the hydrolysis method according to the invention, new, non-bitter hydrolysates can be made available to be used in infant and clinical nutrition, in therapeutic diets as well as in consumer diets and sport nutrition.

Other benefits, not directly related to suppressing bitter tastes include the incubation of the enzyme with food proteins to reduce their allergenicity. Several food proteins contain highly allergenic subfractions, such as wheat gluten that contains prolamines with proline-rich peptide sequences. These proteins can be subjected to the new enzyme to alleviate their antigenicity.

The use of the proline-specific endoprotease is to generate peptides having a carboxyterminal proline residue. Such peptides are desirable additions to various food or nutraceutical products as they have been implicated in anorectic action, in fibrinolytic and antithrombotic and antihypertensive effects, in protection of the gastric mucosa as well as the prevention of rheumatoid arthritis.

In most of these new applications the proline-specific endoprotease should preferably exhibit an activity spectrum with an acidic pH optimum.

To overcome the above-mentioned problems, the invention demonstrates that the activity of an isolated, purified proline-specific endoprotease alone, i.e. without the substantial concomitant or subsequent activity of an exoproteolytic enzyme, is sufficient for significantly debittering a protein hydrolysate. Therefore the proline-specific endoprotease may comprise at least 5 units per gram protein of the enzyme preparation of the invention, preferably 10 u/g, more preferably 25 u/g and even more preferably 50 u/g. Moreover, studies conducted in accordance with the invention demonstrate that the activity of an

Isolated, purified proline-specific endoprotease alone, meaning without the concomitant or subsequent activity of an exoproteolytic enzyme, is sufficient to significantly decrease the overall immunogenicity level of protein hydrolysates, as well as to significantly increase their overall solubility under acidic conditions. The hydrolysates produced according to the invention are enriched in peptides having a carboxy terminal proline residue.

An embodiment of the present invention provides the use of a proline-specific endoprotease, preferably isolated and/or purified, for the high yield production of protein hydrolysates having substantially low bitterness and low allergenic properties without the concomitant production of substantial levels of free amino acids in combination with a DPAP and/or TPAP. All the enzymes may be added at the same time to the substrate or the enzymatic process can be performed in two phase, first the PSE hydrolysis followed by the DPAP and/or TPAP hydrolysis.

Di- and tripeptidyl peptidases present the enzymes of choice for preparing easily assimilable protein hydrolysates. Not only can the peptides formed be directly translocated over the wall of the small intestine but, due to their small size these peptides combine a good water solubility with a lack any allergenic potential. Moreover, vulnerable but indispensible amino acids like glutamine, cysteine and tyrosine are much more stable if present in the form of tripeptides rather than free amino acids. Thus, upon digesting selected proteinaceous substrates with a suitable endoprotease in combination with a tripeptidyl peptidase, hydrolysates are formed in which selected amino acid residues are present in a stable and yet easily assimilable form. Conceivable products that can be conveniently produced using the enzyme mixture according to the invention are easily assimilable gluten hydrolysates supplying high levels of glutamine as well as keratine hydrolysates supplying high levels of cysteine. Likewise hydrolysates containing tripeptides exerting an enhanced modulating, regulatory or hormone-like activity as the result of their increased stability, for example tripeptides rich in proline or glycine residues, could be formed upon the digestion of substrates like gelatin or casein or maize protein. Because of the optimal size and enhanced stability of the peptides present in these hydrolysates, peroral uptake is likely to result in relatively high tripeptide levels in the blood circulation so that the concept of true nutraceuticals comes within reach. Enhanced effects may be

attainable by minor chemical conversions of the peptides formed, e.g. cyclisation of peptides containing proline residues.

The process of the invention is suitable for preparing hydrolysates of various protein fractions. In particular, a protein substrate, such as a milk protein, may be incubated with an isolated, purified proline-specific endoprotease and a DPAP and/or TPAP to produce a protein hydrolysate enriched in peptide fragments having a carboxy terminal proline.

The average length of the peptides in the hydrolysates is in general from 3 to 9 amino acids, preferably from 2 to 6 amino, more preferably from 2 to 4 amino acids.

By peptides or peptide fragments it is meant peptides with molecular masses from 200 to 2000 Dalton. These peptides can be analysed according to the LC/MC analysis as described the "Materials and Methods" section.

In general in the production of the protein hydrolysates of the invention protein substrate is substantially hydrolysed, preferably at least 20% (w/w) of the protein substrate is converted into peptides having molecular masses from 200 to 2000 Dalton. More preferably from 30 to 90% (w/w) and even more preferably from 40 to 80% (w/w) of the protein substrate is converted into such peptides.

Another embodiment of the invention is a protein hydrolysate enriched with a relatively high content of peptides having proline as the carboxy terminal amino acid residue. Since enzyme preparations typically utilized in the genesis of protein hydrolysates are not capable of generating peptides bearing proline residues at carboxy terminii, protein hydrolysates that are relatively rich in such peptides are desired.

Substrates for hydrolysis by an enzyme mixture of the invention include whole milk, skimmed milk, acid casein, rennet casein, acid whey products or cheese whey products. Quite surprisingly the *Aspergillus* derived proline specific endoprotease does not only cleave at the carboxy-terminal side of proline residues but also at the carboxy-terminal side of hydroxyproline residues which makes other, collagen based animal proteins such as gelatine as well as bones or fish-bones containing residual meat, interesting substrates for the enzyme. Moreover, vegetable substrates like wheat gluten and protein fractions obtained from, for example, soy, rice or corn are suitable substrates. Milk protein hydrolysates produced according to the invention may be used with or without additional filtration or purification steps in various speciality foods such as hypoallergenic hydrolysates for infant nutrition, basic hydrolysates for enteral and dietetic nutrition, as well as protein

concentrates for various forms of health food. Thus, protein hydrolysates of the invention may be used to produce foodstuffs having low antigenicity, such as infant formula or requiring facilitated gastro-intestinal uptake, such as various medical or health related products. In addition, enzyme preparations according to the invention may be used to reduce bitterness in foods flavored by at least one protein hydrolysate, even when the protein hydrolysate is present in large amounts. For example, foods may comprise between 5% and 10% (w/v) of a protein hydrolysate and still have their bitterness reduced using an enzyme preparation of the invention.

The present invention preferably uses an isolated or purified proline-specific endoprotease with an acidic pH optimum in a combination with one or more isolated di and/or tripeptidyl peptidases exhibiting acid pH optima for the preparation of a protein hydrolysate for various food applications. Such an isolated, purified proline-specific endoprotease is defined to have at least 10 units of proline specific endoprotease activity per gram of proteinaceous material. These units should be measured using the synthetic peptide Z-Gly-Pro-pNA (Bachem, Switserland) at 37 degrees C and pH 5 in case the pH optimum of the proline-specific endoprotease is below pH 6, for example in case of *Aspergillus niger* proline specific endo protease or else the units should be measured at pH = 7, as specified in the Materials and Methods section. Such a di or tripeptidyl peptidase is defined as an enzyme which is able to cut di or tripeptide from a suitable protein. This enzyme mixture overcomes a number of disadvantages of enzyme mixtures previously known in the art. Most importantly, the isolated, purified proline-specific endoprotease is key in the production of hydrolysates which combine a low allergenic potential, a high yield and a low bitterness profile The isolated di or tripeptidyl peptidases are key in the generation of easily assimilable peptides without any allergenic potential and a specific, preferred amino acid composition. Moreover, the hydrolysates produced with enzyme mixture comprising this proline-specific endoprotease are relatively stable in the body, exhibit a surprising shelf stability upon their incorporation in acid products and contain very low levels of free amino acids, such that minimal off-tastes are generated during heating steps, such as spray drying or product sterilisation. Hydrolysates according to the invention will contain less than 900 micromoles of free amino acids per gram dry weight, preferably less than 300 micromoles of free amino acids per gram dry weight, more preferably less than 150 micromoles of free

amino acids per gram dry weight, and even more preferably less than 50 micromoles per gram dry weight.

Materials and Methods

Sodium caseinate containing 90% protein was obtained from DMV International (The Netherlands). Subtilisin from B.licheniformis (Delvolase®, 560 000 DU per gram) was obtained from DSM Food Specialities (Seclin, France). Thermolysin (Thermoase; a heat stable metallo-endoprotease from Bacillus thermoproteolyticus Rokko with an activity of 14000 PU/ mg) was obtained from Daiwa Kasei, Osaka, Japan).

The enzymatic activity of proline specific endoproteases exhibiting pH optima above pH 6.0 are tested according to T.Diefenthal and H.Dargatz (World Journal of Microbiology &Biotechnology 11, 209-212 (1995)) on Z-Gly-Pro-pNA 0.26 mM in phosphate buffer 0.1M pH 7.0 at 25°C. pH 7.0. The product was monitored spectrophotometrically at 410 nm.Proline specific endoproteases from *Aspergillus* was measured according to the method described in Japanese patent JP5015314 with minor modifications. In brief the enzymatic activity is tested on Z-Gly-Pro-pNA at 37 degrees C in a citrate/disodium phosphate buffer pH 5. pH 5.0 is chosen because in this test the pH optimum of the enzyme is below pH 6. The reaction product was also monitored spectrophotometrically at 410 nM. . The activity of the purified tripeptidyl aminopeptidase (TPAP) as over produced by *A. niger* was measured in a similar way. However, in this case the synthetic substrate Ala-Ala-Phe-pNA (Bachem, Switserland) was used in an incubation in 0.1 mol/litre citrate buffer at pH 4.0 and 60 degrees C. The purified TPAP had an activity of 8 units/ml.

A unit is defined as the quantity of enzyme that provokes the release of 1 µmol of p nitroanilide per minute under these conditions.

The Degree of Hydrolysis (DH) as obtained during incubation with the various proteolytic mixtures was monitored using a rapid OPA test (JFS, Vol 66, NO 5, 2001).

Sensoric evaluation of the protein hydrolysates formed was carried out by an independent institute availing of a panel trained in detecting and ranking various levels of bitterness. During the sssions the taste trials were performed 'blind' and bitterness was scored on a scale from 0 (none)- 4 (very bitter). Panel members were trained with quinine sulphate with the following solutions:

15 ppm quinine sulphate > Intensity bitter = 1
20 ppm quinine sulphate > Intensity bitter = 2
30 ppm quinine sulphate > Intensity bitter = 3
50 ppm quinine sulphate > Intensity bitter = 4

LC/MS analysis.

HPLC (high performance liquid chromatography) using a Qtof-2 (Micromass, Manchester, UK) mass spectrometer was used to separate the peptides formed during digestion with trypsin. 5 microliter of the peptide solution was trapped on a micro-precolumn, C18, 5*0.3 mm (MCA30-05-C18, LC Packings, Amsterdam, Netherlands) using Milli Q water containing 0.1 % of formic acid at a flow-rate of 20 microliter/min. The peptides were then eluted from the precolumn, using a fast gradient of 0.1% formic acid in Milli Q water (Millipore, Bedford, MA, USA; Solution A) and 0.1% formic acid in acetonitrile (Solution B). The gradient started at 100% of Solution A and increased to 60% of solution B in 20 minutes and was kept at the latter ratio for another 5 minutes. The flow rate used during elution of the peptides was 200 nl/min. Using LC/MS/MS analysis partial amino acid sequences of the A. niger proline-specific endopeptidase could be determined, by de novo sequencing of suitable peptides.

HPLC using an ion trap mass spectrometer (Thermoquest®, Breda, the Netherlands) coupled to a P4000 pump (Thermoquest®, Breda, the Netherlands) was used in characterising the enzymatic protein hydrolysates produced by the inventive enzyme mixture. The peptides formed were separated using a PEPMAP C18 300A (MIC-15-03-C18-PM, LC Packings, Amsterdam, The Netherlands) column in combination with a gradient of 0.1% formic acid + 1 mM nonafluoropentaoic acid (NFPA) in Milli Q water (Millipore, Bedford, MA, USA; Solution A) and 0.1% formic acid in acetonitrile (Solution B) for elution. The gradient started at 100% of Solution A and increased to 40% of solution B in 140 minutes and was kept at the latter ratio for another 5 minutes. The injection volume used was 50 microliters, the flow rate was 50 microliter per minute and the column temperature was maintained at 30°C. The protein concentration of the injected sample was approx. 50 micrograms/milliliter.

Detailed information on the individual peptides was obtained by using the "scan dependent" MS/MS algorithm which is a characteristic algorithm for an ion trap mass spectrometer.

Full scan analysis was followed by zoom scan analysis for the determination of the charge state of the most intense ion in the full scan mass range. Subsequent MS/MS analysis of the latter ion resulted in partial peptide sequence information, which could be used for database searching using the SEQUEST application from Xcalibur Bioworks (Thermoquest®, Breda, The Netherlands). Databanks used were extracted from the OWL.fasta databank, available at the NCBI (National Centre for Biotechnology informatics), containing the proteins of interest for the application used. In those experiments in which well characterized protein substrates such as whey proteins or caseins were measured, the precision of the analysis technique was increased by omitting those MS/MS spectra with a sequence fit of less than 50%.

By using different inventive enzyme mixtures the mass range of the peptides formed starts at di- and tripeptides. By using the volatile ion-pairing reagent NFPA in combination with reversed phase liquid chromatography also smaller and more hydrophilic peptides can be monitored ending up with a mass ranging from approx. 200 to 2000 Daltons, considered suitable for further analysis by MS sequencing.

Angiotensin (M=1295.6) was used to tune for optimal sensitivity in MS mode and for optimal fragmentation in MS/MS mode, performing constant infusion of 60 mg/ml, resulting in mainly doubly and triply charged species in MS mode, and an optimal collision energy of about 35 % in MS/MS mode.

LC/MS analysis of infant formulae and commercial protein hydrolysates.

Prior to LC/MS fatty material had to be removed from the infant formulae. To that end the complete nutrition samples (13.5 g powder in 100 ml MilliQ water) were extracted 3 times with 30 ml hexane. Small amounts of NaCl were added to improve separation of the solvent layers. Then 5 ml of the water layer was obtained and freeze dried. Prior to analysis the sample was redissolved in 25 ml of MilliQ water, centrifugated 2 times (at 13000 rpm) and filtered through a 0.22 µm filter. From pure hydrolysated samples, 400 mg was dissolved in 100 ml MilliQ water, centrifugated 2 times (at 13000 rpm) and filtered through a 0.22 µm filter. To characterise the peptides present in the commercial protein hydrolysates,

the same strategy was followed as described above for the enzymatic hydrolysates formed by the inventive enzyme mixture i.e. the filtered hydrolysate was applied to the HPLC column and individual peptides with molecular masses between 200 and 2000 daltons were further characterised by the MS/MS analysis.

Determination of the molar fraction of peptides (%) carrying a carboxyterminal proline.

LC/MS/MS can be used for the analysis of the C-terminus of a peptide. With an algorithm in which the peptide's molecular mass (analyzed with LC/MS) and its (partial) amino acid sequence (analyzed with LC/MS/MS) are linked with automatic search procedures within protein databanks, complex peptide mixtures can be analyzed. These options have enabled us to quantify the incidence of peptides carrying a carboxy terminal proline residue.

To determine in a protein hydrolysate the molar fraction of peptides carrying a carboxyterminal proline, individual peptide peaks eluting from the PEPMAP column are selected and partial carboxyterminal amino acid sequences are determined using the techniques specified above. Analysis of at least 20, preferably at least 30 and more preferably between 40 to 60, for example 50 of the most abundant, randomly choosen peptides thus provides insight in the frequency in which peptides carrying a proline residue at the carboxyterminus of the peptide occur. The quotient of the number of peptides found to carry a carboxyterminal proline residue times 100 and the total number of peptides analysed thus provides the molar fraction of peptides (%) carrying a carboxyterminal proline.

Determination of the molar fraction (%) of proline in the protein substrate used to generate the hydrolysate.

Fatty material as can occur in infant formulae products was first removed by hexane extraction as detailed in the paragraph describing LC/MS analysis of infant formulae and commercial protein hydrolysates. Acid hydrolysis of the protein substrate to convert the proteins present into free amino acids, was achieved by making a suspension of 100 milligrams of proteinaceous material in 2 milliliters 6 N HCl. Acid hydrolysis was carried out for 22 hours at 112 degrees C in an oxygen free atmosphere. After centrifugation the

supernatant was diluted 10 times in dilute HCl. After this hydrolysis the amino acids were derivatised and analysed according to the Picotag method as specified in the operators manual of the Amino Acid Analysis System of Waters (Milford MA, USA). The level of proline present was quantitated using HPLC methods. To determine the molar fraction (%) of proline in the sample, the micromoles of proline present times 100 were divided by the sum of the micromoles of all amino acids present in the sample analysed. Since during acid hydrolysis Trp and Cys are destroyed, these two amino acids are not included in this sum of the micromoles of all amino acids.

Determination of the free amino acid levels in protein hydrolysates or infant formulae.

A precisely weighed sample of the proteinaceous material was dissolved in dilute acid and precipitates were removed by centrifugation in an Eppendorf centrifuge. Amino acid analysis was carried out on the clear supernatant according to the PicoTag method as specified in the operators manual of the Amino Acid Analysis System of Waters (Milford MA, USA). To that end a suitable sample was obtained from the liquid, added to dilute acid and homogenized. From the latter solution a new sample was taken, dried and derivatised using phenylisothiocyanate. The various derivatised amino acids present were quantitated using HPLC methods and added up to calculate the total level of free amino acids in the weighed sample.

To relate this total level of free amino acids in the sample to the total level of amino acids that can be liberated from this sample, the sample is also subjected to acid hydrolysis followed by a quantification of the total free amino acids present as detailed above.

Examples

Example 1

Properties of the tripeptidylpeptidase encoded by gene 12 of *Aspergillus niger*.

The enzyme encoded by gene 12 (described in our copending application PCT/EP02/01984) was overproduced in an *A. niger* host cell and chromatographically purified. Purification was carried out on a Resource Q column in 50 millimol/liter acetate pH

4.5. Elution by increasing the NaCl concentration yielded the enzyme in a sharp activity peak. Activity was measured by incubation with the synthetic peptide Ala-Ala-Phe-pNA. The solution with the purified enzyme contained 8 units/ml if tested on the synthetic tripeptide Ala-Ala-Phe-pNA at pH 4.0 and 60 degrees C (see Materials & Methods section).

In a first experiment the pure enzyme was incubated at pH 5 and 50 degrees C with two different synthetic chromogenic substrates i.e. Ala-Ala-Phe-pNA and Ala-Phe-pNA (both from Bachem, Switserland). Stock solutions of these peptides were made in DMSO which were then diluted 100 x in the desired aqueous buffer. The incubation with the Ala-Ala-Phe-pNA substrate led to a significant increase of the absorbance at 410 nm whereas the incubation with Ala-Phe-pNA did not. This observation clearly demonstrates that this tripeptidylpeptidases can cleave off tripeptides only and does not exhibit aminopeptidase activity that can lead to an undesirable increase of free amino acids.

In a second experiment the preferred stability characteristics of the enzyme encoded by gene 12 was demonstrated. Four samples of the purified enzyme were incubated at pH 5 for one hour at 0, 40, 50 and 60 degrees C respectively. Then to each enzyme sample the above mentioned Ala-Ala-Phe-pNA substrate was added and the enzymatic activity in each heated sample was determined by measuring the increase in absorbance at 410 nm. Whereas the 0 degrees C sample showed 100% activity, the 40 degrees sample showed 96% residual activity, the 50 degrees sample 92% residual activity and the 60 degrees sample 88% residual activity. These data confirm the surprising stability of this *Aspergillus* tripeptidylpeptidase under processing conditions preferred by the food industry.

Finally an impression of the cleavage preferences of the current tripeptidylpeptidase was obtained. To this end an incubation was carried out with the synthetic peptides Ala-Ala-Phe-pNA, Ala-Ala-Ala-pNA and Ala-Ala-Pro-pNA. The three peptides were dissolved in DMSO in 150 mM concentration. The reaction was performed in citrate buffer (0.1 M citrate) pH 4.0 and at 60 °C.

To the cuvette 940 µL of buffer, 50 µL enzyme sample and 10 µL substrate were added and after stirring the reaction was measured kinetically at 405 nm for 10 min. The enzyme was tested in different dilutions.

In order to calculate the specific activity the protein concentration of the enzyme solution was determined spectrophotometrically at 280 nm using a molar extinction coefficient of 1.21 for 1 g/L (based on Trp and Tyr content in the enzyme molecule)

substrates	dilution	U/mL	Specific activity U/mg
Ala-Ala-Phe-pNA	1:50	7.81 – 8.95	2.3
Ala-Ala-Ala-pNA	1:50 - 1:200	76.2 – 81.5	21.8
Ala-Ala-Pro-pNA	non diluted	0.0	0.0

Upon comparison of the absorbances at 410 nm it became clear that the enzyme shows a clear preference for cleaving the Ala-Ala-Ala-pNA substrate. Ala-Ala-Phe-pNA was also cleaved but at a significant lower rate. No activity could be recorded towards the Ala-Ala-Pro-pNA substrate. The latter observation clearly demonstrates that the combination with a proline-specific endonuclease is preferred to convert protein substrates rich in proline residues into readily assimilable, degradation resistant di-and tripeptides with carboxyterminal proline residues.

Example 2

Casein hydrolysates subjected to a proline-specific endoprotease in combination with a tripeptidylaminopeptidase are non-bitter and contain a high proportion of tripeptides having carboxyterminal proline residues.

A 6% (w/w on protein) casein solution was prepared by dissolving sodium caseinate in water. After adjustment of the pH to 8.0 by NaOH, the serine protease Delvolase was added to a concentration of 4% (v/v) and the mixture was incubated for 2.5 hours at 60 degrees C under non-pH-stat conditions. Then the reaction was stopped by lowering the pH to 5.0 using lactic acid followed by a heat treatment of 10 minutes at 90 degrees C. The solution was cooled down to 50 degrees C and two samples were taken. The first sample (Sample A) served as a reference characterizing the material that has been subjected to the

action of a broad spectrum serine protease only. The second sample was used for subsequent incubations with EndoPro and finally TPAP. The incubation with EndoPro was carried out by adding a chromatographically purified solution of the overproduced proline specific endoprotease from *A. niger* in a concentration of 2 units/ gram protein (see our copending application PCT/EP01/14480). After incubating for 16 hours at 50 degrees C under non-pH-stat conditions the EndoPro enzyme was inactivated by another heat treatment to yield Sample B.

In this stage Samples A and B were sensorically evaluated by a trained panel. The two samples were tasted "blind" and then scored on a scale from 0 (non bitter) to 4 (very bitter) as described in the Materials & Methods section. Sample A was unanimously scored as "very bitter", Sample B was unanimously scored as "non bitter". This outcome confirmed the surprising debittering capacity of the EndoPro enzyme once more.

Part of Sample B was then incubated with 20 units of chromatographically purified TPAP per gram of casein protein during 5 hours at pH 4.0 and 60 degrees C. Like before the enzyme reaction was terminated by heating of the solution for 10 minutes at 95 degrees C to yield Sample C.

Samples A, B and C were then subjected to LC/MS analysis (see Materials & Methods section) to determine the size distribution of major peptides present. From all hydrolysates at least 124 different peptides were analysed. The data obtained are shown underneath.

Enzymes used to prepare casein hydrolysate	Heptapeptides or smaller (molar% of all peptides detected)	Di + tripeptides (molar% of all peptides detected)	Tripeptides having carboxyterminal proline residues (molar% of all tripeptides detected)
Subtilisin ("Delvolase")	68	15	0
+ EndoPro (PCT/EP02/01984)	65	17	26
+EndoPro+TPAP (Example 1)	76	21	38

Combining the results of the sensory evaluation and the LC/MS analysis, it is clear that an incubation with both EndoPro and TPAP (i.e. after an incubation with subtilisin) yields a superior product in terms of bitterness (a casein hydrolysate shows no bitterness after incubation with EndoPro) , allergenicity (peptides smaller than 8 amino acid residues) and content of potentially bioactive peptides (tripeptides resisting proteolytic degradation because of their carboxyterminal proline residue).

Example 3

Frequency of di and tripeptides having carboxyterminal proline residues in a commercial casein based infant formula product.

Among the various infant formula products tested (see Example 6 in our copending application PCT/EP01/14480) Nutramigen (Mead Johnson, containing 14 grams of casein hydrolysate per 100 gram powder) contains the highest (i.e. 22%) molar fraction of peptides carrying C-terminal proline. In the present Example we show the results of a LC/MS analysis of this hydrolysate with a focus on its content in di and tripeptides and the frequency of such peptides having carboxyterminal proline residues.

Prior to LC/MS analysis the fatty material present in infant formulae had to be removed. As specified in the Materials & Methods section this was carried out by a hexane extraction. The aqueous phase thus obtained was centrifuged, filtered and then subjected to LC/MS analysis to characterize the various peptides present.

According to the results obtained, the molar fraction of casein derived di- to heptapeptides as present in Nutramigen accounts for 83% of all peptides detected. Furthermore the molar fraction of di + tripeptides as present amongst all peptides detected in Nutramigen could be shown to amount to 18 %. Among the tripeptides identified, a molar fraction of 23 % could be shown to have a carboxyterminal proline residue.

Despite the fact that the protein hydrolysate used represents a product which has probably been highly purified and selectively enriched by a number of techniques such as

ultrafiltration and chromatography, the hydrolysate exhibits a low level of carboxyterminal proline residues which implies considerable bitterness and a limited fraction of protease resistant tripeptides only.

CLAIMS

1. A protein hydrolysate which is rich in di and/or tripeptides whereby the di- and/or tripeptides are rich in proline at one end of the peptide.

2. A protein hydrolysate according to claim 1 wherein at least 20molar%, preferably at least 25molar%, more preferably at least 30molar% of the hydrolysate is present as di and/or tripeptide.

3. A protein hydrolysate according to claim 1 or 2 wherein at least 20%, preferably at least 30%, more preferably at least 40% of the proline present in the starting protein is present in the di and/or tripeptides .

4. A protein hydrolysate according to claims 1 to 3 wherein 30% of the tripeptides, preferably 35% of the trieptide have a carboxy terminal proline.

5. A protein hydrolysate according to claims 1 to 4 wherein at least 70 molar% of the peptides, preferably at least 75 molar% of the pepetides contain 2 to 7 amino acid residues (dipeptide to heptapeptide).

6. A process to produce the protein hydrolysate according to claim 1 to 5 whereby a protein substrate is contacted to
- a proline specific endoprotease (PSE); and
- a dipeptidase (DPAP) and/or tripeptidase (TPAP).

7. A process according to claim 6 whereby the protein substrate is contacted with an endoprotease, preferably a serine protease and/or a metallo endoprotease before, during or after the protein substrate is contacted with PSE.

8. Use of protein hydrolysate according to claims 1 to 5 for mammalian, preferably human, consumption.

ABSTRACT

The present invention describes a protein hydrolysate which is rich in di and/or tripeptides whereby the di- and/or tripeptides are rich in proline at one end of the peptide.